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Short communication

Huperzia quadrifariata and *Huperzia reflexa* alkaloids inhibit acetylcholinesterase activity *in vivo* in mice brainE.L. Konrath^{a,*}, B.M. Neves^a, C.dos S. Passos^a, P.S. Lunardi^b, M.G. Ortega^c, J.L. Cabrera^c, C.A. Gonçalves^b, A.T. Henriques^a^a Programa de Pós Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil^b Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil^c Farmacognosia, Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, IMBIV (Instituto Multidisciplinario de Biología Vegetal, UNC-CONICET), Córdoba, Argentina

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ABSTRACT

Huperzine A, a *Lycopodium* alkaloid produced by Chinese folk herb *Huperzia serrata* (Lycopodiaceae), has been shown to be a promising agent for the treatment of Alzheimer's disease due to its potent acetylcholinesterase (AChE) activity, as well its efficacy in the treatment of memory of aged patients. Thus, the effects of two *Huperzia* species of habitats in Brazil (*H. quadrifariata* and *H. reflexa*) with described *in vitro* AChE inhibition activities were studied and their effects on mice brain AChE inhibition were determined after a single intraperitoneal (i.p.) injection. The alkaloid extracts were administered to mice in various doses (10, 1 and 0.5 mg/kg) and acetylcholinesterase activity was measured *post mortem* in two brain areas using the Ellman's colorimetric method. The AChE activity was found to be significantly reduced in both the cortex and hippocampus, although this activity was less potent than that of reference inhibitor huperzine A (0.5 mg/kg). Thus, it appears that *H. quadrifariata* and *H. reflexa* alkaloid extracts, shown to inhibit acetylcholinesterase *in vitro*, also have very potent *in vivo* effects, suggesting that the *Huperzia* species may still constitute a promising source of compounds with pharmaceutical interest for Alzheimer's disease.

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Introduction

Cholinesterase inhibitors are currently still the only approved drugs for the treatment of patients with mild to moderately severe Alzheimer's disease, a neurodegenerative disorder with progressive deficiency of memory and cognitive function (Bartus et al. 1982; Cummings 2004). The cholinergic hypothesis postulates that memory impairment in patients with Alzheimer's disease results from a deficit in cholinergic function in the brain. The most important changes observed are a decrease in the acetylcholine neurotransmitter, as well in levels of the respective enzymes that synthesize and degrade acetylcholine, choline acetyltransferase and acetylcholinesterase (AChE) (Perry et al. 1977; Bowen et al. 1982). As such, AChE inhibitors can restore the levels of acetylcholine by inhibiting AChE; however none of these agents have the ability to stop disease progression.

Huperzine A, an alkaloid isolated from the Chinese herb *Huperzia serrata* (Lycopodiaceae), is a reversible, potent and selective AChE inhibitor with a longer duration of action and the ability to cross the

blood–brain barrier. This compound induces significant improvements in the memory of aged patients with Alzheimer's disease with fewer noticeable side effects (Tang et al. 1999; Tang and Han 1999). As such, in an attempt to find other AChE inhibitors of plant origin within the family Lycopodiaceae, we have recently screened alkaloids extracts from four *Huperzia* and one *Lycopodiella* species from Brazil for their *in vitro* enzymatic inhibitory effects (Konrath et al., 2012b) and also the *in vitro* and *ex vivo* effects of *Lycopodium clavatum* and *L. thyoides* on AChE (Konrath et al. 2012a). Among these, the extracts of *Huperzia reflexa* (Lam.) Trevis and *Huperzia quadrifariata* (Bory) Rothn. showed the greatest anticholinesterase activity with a selectivity for the true acetylcholinesterase; with IC₅₀ = 0.11 and 2.0 µg/ml, respectively. These effects are attributed to the *Lycopodium* alkaloids present in the extracts, a group of compounds obtained from club mosses belonging to the Lycopodiaceae family, found to possess potent anticholinesterase activity (Liu et al. 1986; Liu and Huang 1994; Ma and Gang 2004). Within this class of plant metabolites, only a few of these have been demonstrated to be acetylcholinesterase inhibitors, including huperzine A, which also has a reported effect on improving memory and learning (Vincent et al. 1987; Zhang and Wang 1990).

Based on this evidence, the aim of the present study was to conduct further investigations in order to better characterize

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the anticholinesterase effects of *H. quadrifariata* and *H. reflexa*, using an *in vivo* model, in an attempt to correlate the results obtained, together with the chemical characterization of the above-mentioned extracts. The findings from the present study suggest that *H. quadrifariata* and *H. reflexa* may be sources of candidate compounds for further research for the treatment of Alzheimer's disease.

Materials and methods

Chemicals

Acetylthiocholine iodide, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and huperzine A were obtained from Sigma Chemical Co (USA). Dimethyl sulfoxide (DMSO) and the buffer salts were purchased from Merck (Germany). Water was treated in a Milli-Q (Millipore, Bedford, Massachusetts) water purification system. All other reagents and organic solvents were of the highest grade available. The extracts were lyophilized and initially dissolved in DMSO; solutions were then diluted in saline immediately before each administration.

Plant material

Aerial parts of *Huperzia quadrifariata* (0.58 kg) and *Huperzia reflexa* (0.092 kg) were collected in Rio Grande do Sul, Brazil, in April 2009 and July 2008, respectively. The material was identified and authenticated by Prof. Dr. Sérgio de Loreto Bordignon of the Centro Universitário La Salle. Voucher specimens (HAS 47475 and 45877) have been deposited in the Fundação Zoobotânica do Rio Grande do Sul Herbarium for future reference.

Preparation of extracts and chemical composition

Air dried aerial parts were ground and separately defatted with *n*-hexane using a Soxhlet extractor for 2 days. The remaining plant materials were subsequently extracted with EtOH by the same procedure, until the eluate was negative to Dragendorff's reagent. The EtOH extracts were concentrated under vacuum; the dry residues were suspended in 5% HCl and washed with CH₂Cl₂. The aqueous layers thus obtained, were basified with NH₄OH (pH 11) and partitioned with CH₂Cl₂, affording, after filtration under Na₂SO₄, crude alkaloid extracts (AE). *Huperzia quadrifariata* AE (HQAE) and *Huperzia reflexa* AE (HRAE) were then submitted to a GC–MS analysis on a Perkin–Elmer Qmass-910 apparatus with a SE 30 capillary column of 30 m in length in order to verify the chemical composition. The injection volume was 0.5 µl with He as carrier, with the flow rate being 1 ml/min. Temperature program: 140 °C (3 min), 140–250 °C at 5 °C/min, 250 °C (5 min), 250–280 °C at 5 °C/min, 280 °C (5 min). The temperatures of the injector, interface and ion source were 300, 280 and 280 °C respectively. The ionization energy was 70 eV and individual alkaloid identifications were made by comparing breakdown patterns with those found in the literature and with our own library of isolated Lycopodium alkaloids (MacLean 1963; Alam et al. 1964; Sun et al. 1993; Ortega et al. 2004) (Figs. S1 and S2).

Animals and care

Swiss albino male mice of the CF1 strain (25–35 g), obtained from the Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS) were used. Animals were housed in plastic cages and maintained at 22–23 °C under a 12 h light/dark cycle (lights on at 7:00 a.m.) with free access to food and water. All experiments were

conducted blind to the treatment condition of the animals and following the USA National Institute of Health Guidelines for Animal Care and Use and approved by the Animal Care and Ethics Committee of the Universidade Federal do Rio Grande do Sul.

Ex vivo acetylcholinesterase inhibition assay

Groups of six Swiss albino male mice were treated i.p. (0.1 ml/10 g) with different doses of HRAE (10, 1, 0.5 and 0.1 mg/kg), HQAE (10, 1 and 0.5 mg/kg) and huperzine A (0.5 mg/kg). The control animals received the same volume of saline and 20% DMSO (administration vehicle). Mice were sacrificed 60 min later, brains were quickly removed on an ice-cold plate; cortex and hippocampi were dissected out and homogenized in 10 volumes of cold 20 mM phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 × *g* for 10 min and the supernatants were employed as sources of enzyme. All steps were carried out at 4 °C and the total AChE activity was determined using the spectrophotometric method of Ellman et al. (1961). Each brain homogenate preparation was mixed with a buffered solution of Ellman's reagent (10 mM DTNB) and acetylthiocholine at a concentration of 0.8 mM. Hydrolysis rates were measured at 415 nm for 3 min with 30 s intervals, monitored by the formation of the thiolate dianion of DTNB. Percentages of inhibitions were calculated by comparing AChE activity in the aliquots of brain homogenates with activity of mice treated just with DMSO.

Protein assay

Total protein concentrations were determined as described by Lowry et al. (1951), using bovine serum albumin as standard.

Statistics

All assays were performed in triplicate and the mean was used for statistical analysis. Data were analyzed by ANOVA followed by Duncan's multiple range test when the *F* test was significant. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software using a PC-compatible computer.

Results and discussion

After the discovery that the alkaloid huperzine A is a potent acetylcholinesterase inhibitor and, consequently, very important for the symptomatic treatment of Alzheimer's disease, the interest in the isolation and characterization of alkaloids from Lycopodiaceae has increased exponentially (Liu et al. 1986; Ma and Gang 2004). However, due to the low alkaloid content and the very limited distribution of *H. serrata*, other alternative sources are currently under investigation to obtain huperzine A in large quantities. Endophytic fungi associated with *H. serrata* were also found to synthesize this alkaloid, becoming an alternative way to produce huperzine A, thus eliminating the need to harvest and extract wild populations of *Huperzia* (Wang et al. 2011).

In this respect, we initiated the study of some species belonging to this family with habitats in Brazil; the results of our previous studies showed a promising *in vitro* inhibiting effect upon acetylcholinesterase activity (Konrath et al., 2012b), which prompted us to further investigate *in vivo* models in order to better characterize such effects.

Figs. 1 and 2 show the effects of HQAE (10–0.5 mg/kg) and HRAE (10–0.1 mg/kg) on AChE activity in the hippocampus and cortex of mice. The experimental conditions were validated with huperzine A (0.5 mg/kg), which inhibited the enzyme in all brain regions (64% in cortex and 47.5% in hippocampus) (*p* < 0.05). As already reported, DMSO was devoid of effect (*p* > 0.05) (Siqueira et al. 2003), with no

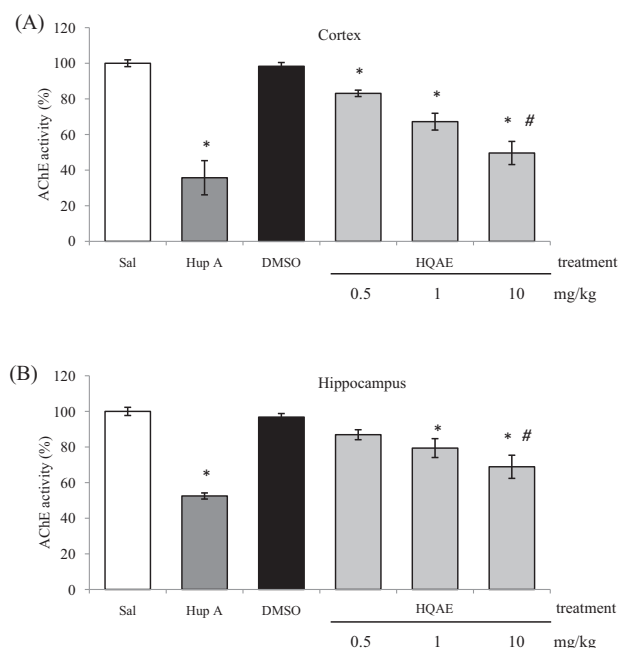


Fig. 1. Effect of the acute administration of the alkaloid extract of *H. quadrifariata*, HQAE (0.5, 1 and 10 mg/kg i.p.) and huperzine A (0.5 mg/kg i.p.), on AChE activity in the mouse cortex and hippocampus. Enzyme activity is expressed as the percentage of control (DMSO-treated mice); all assays were performed in triplicate. Each value represents mean \pm S.E.M. ($n=6$). * $p < 0.05$ vs control (DMSO); # $p < 0.05$ vs previous dose, Duncan's test comparison after ANOVA.

significant differences when compared to saline-treated groups. HQAE inhibited enzyme activity in the cortex, at all doses tested (Fig. 1A), achieving 50.4, 33 and 17% of inhibition ($p < 0.05$), for 10, 1 and 0.5 mg/kg, respectively, while in hippocampus the effect was less marked, with significant values of inhibition of 31 and 20.5%

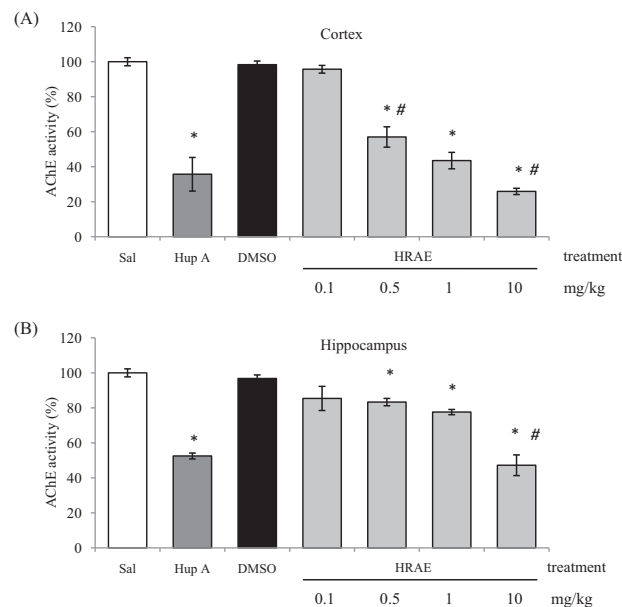
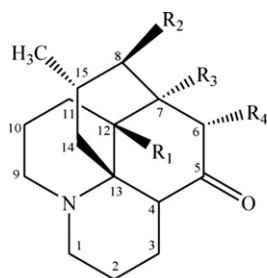


Fig. 2. Effect of the acute administration of the alkaloid extract of *H. reflexa*, HRAE (0.5, 1 and 10 mg/kg i.p.) and huperzine A (0.5 mg/kg i.p.) on AChE activity in the mouse cortex and hippocampus. Enzyme activity is expressed as the percentage of control (DMSO-treated mice); all assays were performed in triplicate. Each value represents mean \pm S.E.M. ($n=6$). * $p < 0.05$ vs control (DMSO); # $p < 0.05$ vs previous dose, Duncan's test comparison after ANOVA.

for 10 and 1 mg/kg (Fig. 1B). The AChE activity was also markedly affected by HRAE administration, with a 74, 56 and 43% of inhibition ($p < 0.05$) in the cortex (Fig. 2A) and 53, 22 and 17% in the hippocampus (Fig. 2B) for 10–0.5 mg/kg, respectively. Only after the administration of the AE at a lower dose (0.1 mg/kg) did the extract show no significant activity on the enzyme in brain struc-



Lycopodium group

$R_1=R_2=R_3=R_4=H$

Lycopodine

$R_1=OH$; $R_2=R_3=R_4=H$

Lycodoline

$R_2=OH$; $R_1=R_3=R_4=H$

Clavolonine

$R_2=OAc$; $R_1=R_3=R_4=H$

Acetylclavolonine

$R_1=R_2=R_3=H$; $R_4=OH$

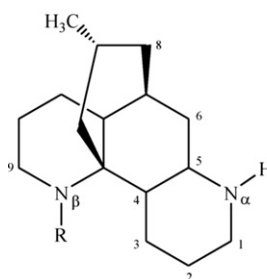
6-hydroxylycopodine

$R_2=R_3=OH$; $R_1=R_4=H$

Sauroine

$R_1=R_2=R_3=R_4=H$; $\Delta^{11,12}$

Anhydrolycodoline



Lycodine group

$\Delta^{N\alpha,1}$; $\Delta^{2,3}$; $\Delta^{4,5}$; $R=H$

Lycodine

$\Delta^{4,5}$; $R=CH_3$; $C_1=O$

α -obscurine

$\Delta^{4,5}$; $R=H$; $C_1=O$

Des-*N*-methyl- α -obscurine

Fig. 3. Structures of the Lycopodium alkaloids identified in *Huperzia quadrifariata* and *Huperzia reflexa* alkaloid extracts by GC/MS.

tures ($p > 0.05$), showing that the alkaloid content in *Huperzia reflexa* possesses a better anticholinesterase inhibitory profile when compared to *Huperzia quadrifariata*.

The results obtained in the current study suggest that one or more alkaloids present in the extracts reach the brain following intraperitoneal administration, crossing the blood–brain barrier and inhibiting cholinesterase in selected brain areas, consistent with evidence of the already described inhibition of the brain enzyme *in vitro*. The profile of inhibition demonstrated that AChE was preferentially affected in mice cortex following the injection of alkaloids. Although spatial learning and memory are closely related to hippocampal function in rodents (Moser et al. 1993), cholinergic deficits in both the hippocampus and cortex may occur in Alzheimer's disease, together with atrophy of surviving cholinergic neurons in the basal forebrain in aged animals (Muir 1997).

Ten *Lycopodium* alkaloids belonging to lycopodane and flabellidane groups were detected in the extracts by means of GC–MS (Fig. 3). Among these, clavolonine, acetylclavolonine, lycopodine, 6-hydroxylycopodine and sauroine were found in HQAE, while the presence of the alkaloids lycopodine, 6-hydroxylycopodine, lycodoline, anhydrolycodoline, α -obscurine, des-*N*-methyl- α -obscurine and lycodine was confirmed in HRAE, along with other alkaloid compounds whose structures have not yet been determined (Konrath et al., 2012b). It has been reported that approximately 200 alkaloids were isolated from different species of this family; some of them exhibit a prominent anticholinesterase activity including huperzine B, *N*-demethylhuperzine and sieboldine A (Shen and Chen 1994; Hirasawa et al. 2003). In this study, we demonstrated that alkaloids present in the extracts manifested AChE inhibitory activity, although they are less potent than huperzine A. However, none of the alkaloids detected in the extracts surprisingly correspond with any known acetylcholinesterase inhibitor within this class of compounds. It should be remembered, though, that active doses of the extracts are likely to be diminished with further purification of active compounds. As such, a bioassay-guided fractionation of the extracts will be conducted in order to identify and isolate the active alkaloid/s, since only a few of these have reported anti-AChE activity, as shown by *in vitro* assays, and none of them have demonstrated activity *in vivo*.

Thus, our study demonstrates that the species, *Huperzia quadrifariata* and *Huperzia reflexa*, appear to demonstrate newly identified and important anticholinesterase activity, corroborating our previous results. Further research is also necessary to define the actual potential of the use of these agents in humans, using other models of Alzheimer's disease. In addition, pharmacological evaluation with isolated alkaloids is in progress in our laboratory.

Conflict of interest

The authors have declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phymed.2012.08.009>.

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